REMARKS

Upon entry of the Amendment, claims 2, 5, 7, 9, 10, 15, 16, 18, 19, 22, and 23 are pending in the application. Claims 1, 24, and 25 are canceled. Claims 2, 5, and 9 are amended. Claims 15, 16, and 18 are allowed.

No new matter is added. Claim 2 is amended from a dependent claim to an independent claim. The amendments to claims 5 and 9 correct minor errors.

These amendments do not raise new issues or require further search. Entry of the Amendment is respectfully requested.

I. Claim Rejections - 35 U.S.C. § 112, first paragraph

(A) Claims 9 and 10 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement.

Claims 9 and 10 are drawn to a process of producing a recombinant polypeptide rather than a method of using the recombinant polypeptide.

Nevertheless, the Examiner asserts that a person skilled in the art cannot use the recombinant polypeptide recited in claim 9 or the recombinant hWAPL protein recited in claim 10. The Examiner asserts that data on the oncogenesis inducing potential of expression of an hWAPL polypeptide in a cell line does not allow a person skilled in the art to predict that the hWAPL polypeptide is expressed in tumor cells or is involved in oncogenesis.

Applicants respectfully traverse for the following reasons.

A person skilled in the art can use the hWAPL protein without undue experimentation, as the specification describes evidence establishing that the hWAPL protein is expressed in tumor cells or is involved in the development of HPV infection cancer. For example, the specification

teaches that the hWAPL protein is expressed in a human cancer cell. Page 45 of the specification describes as follows:

> We have conducted Western blotting analysis for extracts from Saos-2 cell and NIH3T3 cell, using an anti-hWAPL-N antibody and an anti-hWAPL-C antibody, which are antibodies specific to the peptide chain in the human WAPL protein, as explained in Example 6. As shown in FIG. 4, the results have revealed a band of a protein reactive to the antibody with approximately 140 kDa. Thus, it has been confirmed that the human WAPL protein is actually present in the human-derived cell in some extent.

In this regard, Applicants have provided sufficient evidence demonstrating that the human WAPL protein is produced in a human "Saos-2" cell. The Saos-2 cell is a human osteogenic sarcoma cell having an origin established from the primary osteogenic sarcoma of an 11-year old Caucasian woman in 1973. *See, e.g.*, <http://www.dsmz.de/human_and_animal_cell_lines/info.php?dsmz_nr=243&from=cell_line_index&select=search_for_term&term=*&preselect=human> (last visited August 30, 2007). The DSMZ number thereof is ACC 243. *Id.* As a result, the Saos-2 cell possess the features of a cancer cell.

Example 2 of the specification also discloses that the hWAPL mRNA is expressed in cervical and some gastric cancers. Applicants respectfully submit that at least the identification of the mRNA expression reasonably establishes that the hWAPL polypeptide is expressed in tumor cells or is involved in oncogenesis. Probe hybridization or real-time RT-PCR methods are widely used to monitor the expression level of a mRNA. Such a wide-use of probe hybridization is based on the generally accepted knowledge a high level of mRNA would provide for a high level of the protein encoded by the mRNA. Complete control of gene expression at the posttranscriptional level is a ***rare*** exception.

While the Examiner cites Fu et al, Brennaan et al., Zimmer et al., the cited art does not establish that mRNA is an overall unreliable indictor of protein expression. As described above, the norm is that mRNA expression levels correlate with protein expression levels. An entire industry has developed around quantifying mRNA expression in cells, such as with real time RT-PCR, on the basis that relative mRNA levels typically correlate with protein levels. A person skilled in the art would understand that the results disclosed in the cited art are not applicable to all circumstances, such as with hWAPL. It may be that the results disclosed in the cited art may be a condition causing the particular disease or disorder thereof or that the results disclosed in the cited art are caused by the particular disease or disorder thereof.

Brennan *et al.*, cited by the Examiner, states at page 185: "Failure to detect IFNγ and LT in the culture supernatants despite the presence of high mRNA levels was ***unexpected***." (emphasis added). In this regard, Brennan *et al* indicates that a person skilled in the art would normally expect protein expression where the mRNA is also expressed in the cell. There is no basis indicating the hWAPL polypeptide is one of those rare cases in which the expression of hWAPL mRNA is not indicative of expression of a hWAPL polypeptide. There is also no basis indicating that the translation of hWAPL mRNA is selectively blocked from expressing a correlative amount of the hWAPL protein.

Further, the specification provides evidence that that the increased expression levels of hWAPL protein is closely associated with cancerization. Example 7 of the specification demonstrates that over-expression of the hWAPL protein induces chromosome instability in a HeLa cell and that over-expression of the hWAPL protein initiates the induction of multi-nucleation and micronuclei formation. Example 7 describes infecting a HeLa cell with an expression vector for producing a recombinant protein of a hrGFP-fused hWAPL. The

expression of the GFP-hWAPL protein is detected by using the GFP protein as a diffusion partner for monitoring the green fluorescence of the GFP portion of the GFP-hWAPL protein.

Example 8 of the specification demonstrates that over-expression of the HA-tagged hWAPL protein induces cancerization. The Western Blot described therein shows that the HA-tagged hWAPL protein is over-expressed in the cancerized cell thereof. While no oncogenesis was induced with a negative control cell line (i.e., HA-3T3 cell line), oncogenesis was induced with a cell infected with an expression vector for producing a recombinant protein of HA-tagged hWAPL (i.e., HA-hWAPL 3T3 cell line).

The specification provides sufficient guidance for a person skilled in the art to conduct an immunohistochemical analysis that detects increased expression levels of the hWAPL protein. The increased expression levels of the hWAPL protein in turn detects a region forming a tumor. Example 8 of the specification indicates that monoclonal antibodies that recognize hWAPL can be used to detect a region forming a tumor. At a minimum, recombinant hWAPL protein can be used as an antigen to produce monoclonal antibodies that recognize hWAPL protein and can be used to immunohistocehmically identify increased expression levels of the hWAPL protein. Oikawa, *et al.*, "Expression of a Novel Human Gene, *Human Wings Apart*-Like (*h*WAPL), Is Associated with Cervical Carcinogenesis and Tumor Progression," Cancer Research 64, 3545-3549 (May 15, 2004), discloses employing an immunohistochemical analysis for the purpose of monitoring the development of cervical cancer. A copy thereof is provided herewith for the convenience of the Examiner.

(B) Claims 1, 5, 7, 24, and 25 are rejected under 35 U.S.C. § 112, first paragraph, allegedly because the specification, while being enabling for a polynucleotide comprising the nucleotide

sequence of SEQ ID NO:2, is allegedly not enabling for other polynucleotides encoding the polypeptide of SEQ ID NO:1.

Specifically, the Examiner contends that the specification only establishes that a polynucleotide of SEQ ID NO:2 is overexpressed in cancer cells. Further, since the Examiner concludes that the encoded polypeptide has no enabled use (see above), in the Examiner's opinion, other polynucleotides encoding the polypeptide of SEQ ID NO:1 are likewise not enabled.

Without admitting that the rejection is correct, Applicants respectfully submit that claim 5 presently recites that the polynucleotide consists of a nucleotide sequence of SEQ ID NO: 2. In this regard, claim 5 is fully enabled.

Claims 1, 24, and 25 are canceled. Claim 7 depends from claim 5. In this regard, claim 7 is fully enabled for at least the same reasons as claim 5.

II. Claim Rejections - 35 U.S.C. § 112, second paragraph

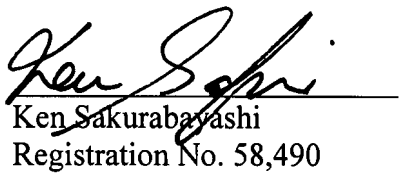
Claims 2, 5, 7, and 25 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

The Examiner asserts that the phrase "is represented by SEQ ID NO:2" is unclear. Claims 2 and 5 are amended to recite a nucleotide sequence "of" SEQ ID NO:2.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Ken Sakurabayashi
Registration No. 58,490

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: September 4, 2007

[CANCER RESEARCH 64, 3545–3549, May 15, 2004]

P. 3545-3549(5)

Expression of a Novel Human Gene, *Human Wings Apart-Like (hWAPL)*, Is Associated with Cervical Carcinogenesis and Tumor Progression

Kosuke Oikawa,[1,3,4] Tetsuya Ohbayashi,[1,3,4] Tohru Kiyono,[5] Hirotaka Nishi,[2] Keiichi Isaka,[2] Akihiro Umezawa,[4,6] Masahiko Kuroda,[1,3,4] and Kiyoshi Mukai[1]

Departments of [1]Pathology and [2]Obstetrics–Gynecology, Tokyo Medical University, Shinjuku-ku, Tokyo; [3]Core Research for Evolutional Science and Technology Research Project, Japan Science and Technology Corp., Kawaguchi-shi, Saitama; [4]Shinanomachi Research Park, Keio University, Shinjuku-ku, Tokyo; [5]Division of Virology, National Cancer Center Research Institute, Chuo-ku, Tokyo; and [6]National Research Institute for Child Health and Development, Setagaya-ku, Tokyo, Japan

ABSTRACT

In *Drosophila melanogaster*, the *wings apart-like* (*wapl*) gene encodes a protein that regulates heterochromatin structure. Here, we characterize a novel human homologue of *wapl* (termed *human WAPL*; *hWAPL*). The *hWAPL* mRNA was predominantly expressed in uterine cervical cancer, with weak expression in all other normal and tumor tissues examined. hWAPL expression in benign epithelia was confined to the basal cell layers, whereas in dysplasias it increasingly appeared in more superficial cell layers and showed a significant correlation with severity of dysplasia. Diffuse hWAPL expression was found in all invasive squamous cell carcinomas examined. In addition, NIH3T3 cells overexpressing hWAPL developed into tumors on injection into nude mice. Furthermore, repression of *hWAPL* expression by RNA interference induced cell death in SiHa cells. These results demonstrate that hWAPL is associated with cell growth, and the *hWAPL* expression may play a significant role in cervical carcinogenesis and tumor progression.

INTRODUCTION

The *wings apart-like* (*wapl*) gene of *Drosophila melanogaster* encodes a protein that regulates heterochromatin structure (1). Mutations of *wapl* prevent the normal close apposition of sister chromatids in heterochromatin regions but do not appear to affect either heterochromatin condensation or chromosomal segregation (1). This evidence suggests that *wapl* is required to hold sister chromatids together in mitotic heterochromatin. *wapl* has also been implicated in both heterochromatin pairing during female meiosis and the modulation of position effect variegation (1). In addition, a *P* element screen of *Drosophila* identified *wapl* as a modifier of chromosome inheritance (2).

Among all varieties of cancer, uterine cervical cancer is unique because of its association with high-risk human papillomavirus (HPV) infection, with strains like HPV-16 and HPV-18. High-risk HPVs encode two oncoproteins, E6 and E7, which subvert crucial cellular regulatory mechanisms that reactivate and maintain DNA synthesis in the host cell. E6 accelerates proteosomal degradation of the p53 tumor suppressor, and E7 inactivates the retinoblastoma protein, interfering with the action of both $p16^{INK4a}$ (3) and the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (4, 5). Both the E6 and E7 high-risk HPV oncoproteins independently induce genomic instability in normal human cells (6, 7). Only a small portion of precursor lesions infected with HPV, however, develops into invasive carcinomas (8). Therefore, additional genetic and microenvironmental factors subsequent to HPV infection are thought to play an important role in the initiation and progression of cervical neoplasia (8–10).

In this study, we describe the isolation and characterization of a novel human *wapl*-related gene termed *human WAPL* (*hWAPL*). We have also demonstrated that *hWAPL* has the characteristics of an oncogene and is associated with uterine cervical cancer.

MATERIALS AND METHODS

cDNA Cloning and Construction of the hWAPL Expression Vector. To isolate the complete *hWAPL* cDNA sequence, we used a human testis Marathon-Ready cDNA kit (Clontech, Palo Alto, CA).

To create an expression vector encoding *hWAPL*, a *Hin*dIII-*Eco*RI cDNA fragment containing the complete coding region of hWAPL was amplified by PCR using the primers 5′-TTAAGCTTTGAAACTGGTGTCAAAATGACATCCAGATT-3′ and 5′-TTGAATTCAAGCAATGTTCCAAATATTCAATCACTCTAGAG-3′ and inserted into the hemagglutinin (HA)-tagged mammalian expression vector, pHM6 (HA-hWAPL; Roche Diagnostics, Mannheim, Germany).

Northern Blot and Quantitative Real-Time PCR Analysis. RNA isolation (11) and Northern blot analysis (11, 12) were performed as described. The 674-bp *Dpn*II fragment of *hWAPL* cDNA was used as a probe and labeled with ^{32}P using the Rediprime II random prime labeling system (Amersham Biosciences, Piscataway, NJ). A human β-actin cDNA control probe (Clontech) was used as a control.

First-strand cDNA synthesis was performed as described (13). Real-time PCR analysis was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) with SYBR Green I (Cambrex, Washington, DC). Real-time PCR used the *hWAPL*-specific primers 5′-GAATTCATAGGCACAGCGCTGAACTGTGTG-3′ and 5′-TTGAATTCCTAGCAATGTTCCAAATATTCA-3′ and β-actin-specific primers 5′-GGGAAATCGTGCGTGACATTAAG-3′ and 5′-TGTGTTGGCGTACAGGTCTTTG-3′. Reaction mixtures were denatured at 95°C for 30 s and then were subjected to 40 PCR cycles at 95°C for 3 s, 68°C for 30 s, and 87°C for 6 s. *hWAPL* mRNA levels were normalized to β-actin signals.

Immunohistochemistry and Immunoblot Analysis. To generate mouse monoclonal antibodies against hWAPL, we immunized mice against a 6 × histidine-tagged hWAPL COOH terminus (amino acids 814-1037) fusion protein. Spleen cells of an immunized mouse were fused with P3UI mouse myeloma cells as described previously (14). Of the 128 hybrids generated, one clone (clone R929) showed exclusive reactivity with hWAPL by ELISA. We used the supernatant of this clone as anti-hWAPL antibody.

Immunohistochemical assays were performed on formalin-fixed, paraffin-embedded sections using Ventana HX System Benchmark (Ventana Medical Systems Inc., Tucson, AZ). Immunohistochemical stains for hWAPL were interpreted semiquantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides as described (9).

Immunoblot analyses were performed as described previously (15). The anti-HA (Roche Diagnostics; 3F10) and monoclonal anti-α-tubulin clone B-5-1-2 (Sigma Chemical Co., St. Louis, MO; T-5168) antibodies were purchased.

Animals and Treatment. BALB/cAJc1-nu female mice (4 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan).

The tumorigeneicity of the stable NIH3T3 transformants overexpressing hWAPL *in vivo* was examined as described previously (16).

Cell Culture and small interfering RNA (siRNA) Transfection. SiHa and NIH3T3 cells were grown in DMEM (Sigma) containing 10% fetal bovine serum at 37°C in a 5% CO_2 environment. For the transfection of siRNA, we

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Note: T. Ohbayashi is currently at the Horizontal Medical Research Organization, Kyoto University Faculty of Medicine, Kyoto, Japan.

Requests for reprints: Masahiko Kuroda, Department of Pathology, Tokyo Medical University, 6-1-1, Shinjuku, Shinjuku-ku, Tokyo, 160-8402, Japan. Fax: 81-3-3352-6335; E-mail: kuroda@tokyo-med.ac.jp.

generated siRNAs using a Silencer siRNA Construction Kit (Ambion, Austin, TX). siRNA transfection was performed in DMEM without serum using Oligofectamine Reagent (Invitrogen Japan, Tokyo, Japan) and Opti-MEM I (Invitrogen Japan).

For cell quantitation, we harvested the cells from the wells of a 12-well plate and resuspended them in 100 μl of PBS. Trypan blue solution (100 μl, 0.4%; Sigma) was added to each sample, and viable cell numbers were quantitated using an erythrometer. The results shown are representative of three independent cell count analyses.

RESULTS

Molecular Cloning of *hWAPL*. To isolate *wapl*-related genes from human cells, we searched DNA databases and identified a cDNA fragment, KIAA0261 (17), and three expressed sequence tag clones, BE410177, BF79516, and BE257022, containing the KIAA0261 sequence. We also performed 5′ rapid amplification of cDNA ends. From these DNA sequences, we cloned and confirmed the full-length coding region sequence of the cDNA containing KIAA0261. We named this gene *hWAPL* (GenBank accession no. AB065003) to reflect its homology to *wapl*. The *hWAPL* gene product shows high sequence similarity in the WAPL-conserved region (amino acids 627-1169, 34% identical and 56% similar) and low similarity throughout the other regions to the *wapl* gene product. Several additional stretches of amino acids are also present in wapl protein (Fig. 1*A*).

High-Level Expression of hWAPL in Human Cervical Cancer. As *wapl* is involved in sister chromatid cohesion, hWAPL may modify chromosomal inheritance. Deregulation of the expression of genes involved in chromosomal inheritance directly induces a variety of disorders associated with aneuploidy, including birth defects and cancer. Northern blot analysis detected *hWAPL* mRNA expression in several invasive cervical cancer samples, examined in tandem with additional human cancers and normal tissues (Fig. 1*B*). We confirmed the *hWAPL* expression in cervical cancers by quantitative real-time PCR analysis of tumor and normal tissue samples. The levels of *hWAPL* mRNA expression in cervical cancers were significantly higher than the levels observed in either normal cervical controls or endometrial, ovarian, breast, lung, stomach, renal, and colon cancers (Fig. 1*C*).

To investigate the connection between hWAPL expression and oncogenesis in cervical malignancies, we examined the expression of hWAPL by immunohistochemistry in a series of clinical samples of the various grades of cervical dysplasia [cervical intraepithelial neoplasia (CIN) I–III] and invasive squamous cell carcinoma. We found nuclear immunostaining for hWAPL in all samples (Fig. 2*A*). hWAPL expression in benign squamous epithelia was confined to the basal and parabasal cell layers. In contrast, hWAPL expression in squamous dysplasia and invasive carcinoma increasingly appeared in the more superficial cell layers and was significantly increased compared with the adjacent benign epithelia ($P = 0.0002$ for CIN I, $P = 0.0003$ for CIN II, $P = 0.0001$ for CIN III, and $P = 0.0001$ for invasive squamous cell carcinoma; Wilcoxon's signed rank test). CIN I and II cases showed hWAPL expression in the basal 50 and 70% of the epithelial thickness, respectively, whereas CIN III and invasive squamous cell carcinoma showed hWAPL expression in the full thickness of the dysplastic epithelia (Fig. 2*A*). Furthermore, the mean hWAPL staining score increased remarkably with increasing grade of dysplasia (Fig. 2*B*). These data strongly suggest that the unscheduled high-level expression of hWAPL may play a significant role in cervical carcinogenesis and tumor progression.

hWAPL Has Oncogenic Characteristics. Because we observed high-level expression of *hWAPL* in tumors, we sought to determine whether hWAPL overexpression promotes tumor development. We transfected NIH3T3 cells with an HA-tagged hWAPL expression vector (HA-hWAPL 3T3) or HA expression vector (HA-3T3). Then, we compared the ability of HA-hWAPL 3T3 with HA-3T3 cells to grow as tumors in nude mice. We injected 10^6 cells into three s.c. sites of each nude mouse. HA-hWAPL 3T3 cells produced tumors in all nude mice within 10 days after injection of cells (100%, $n = 18$; Fig. 3*A*). HA-3T3 failed to produce tumors in any mice (0%, $n = 18$). We confirmed high hWAPL expression levels in the resultant tumors by Western blot analysis (Fig. 3*B*). These results suggest that *hWAPL* has the characteristics of an oncogene.

Repression of hWAPL Expression Induces Cell Death. We examined hWAPL function by suppressing hWAPL expression. Initial attempts to generate a *WAPL*-deficient mouse demonstrated that the loss of *WAPL* was embryonic lethal (data not shown). Therefore, we designed two 21-nucleotide, double-stranded siRNAs, siRNA(I) and siRNA(II), to repress *hWAPL* expression (Refs. 18 and 19; Figs. 1*A*

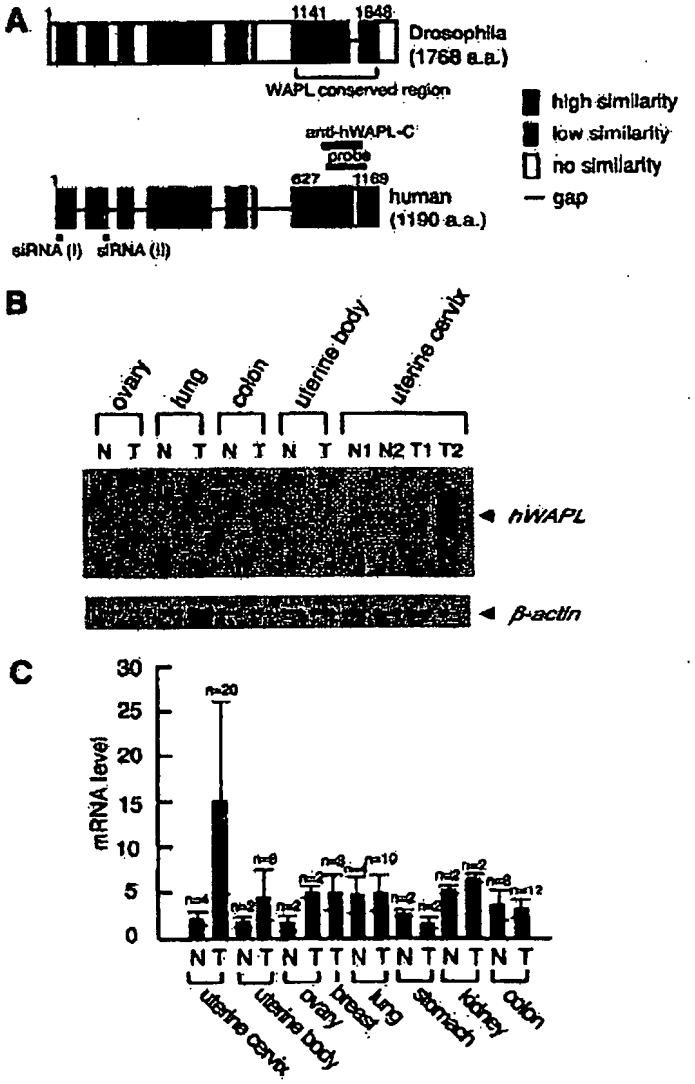

Fig. 1. Structures of wings apart-like (*WAPL*) proteins and *human WAPL* (*hWAPL*) expression in normal and tumor human tissues. *A*, schematic structure of the *hWAPL* and *Drosophila wapl* gene products. The site corresponding to the probe sequence used for Northern blot analysis is indicated by "probe." The antibody recognition site is indicated by "hWAPL-C." The small interfering RNA (*siRNA*) targeting sites are indicated by "siRNA(I)" and "siRNA(II)." *B*, Northern blot analysis of *hWAPL* in several normal (*N*) and tumor (*T*) human tissues. *C*, quantitative real-time PCR analysis demonstrating *hWAPL* mRNA levels in various normal (*N*) and tumor (*T*) human tissues. *Columns*, the means of examined samples. The minimum mRNA expression level was arbitrarily set to 1 in the graphical presentation; all other mRNA signals were normalized to this value. *Bars*, SD.

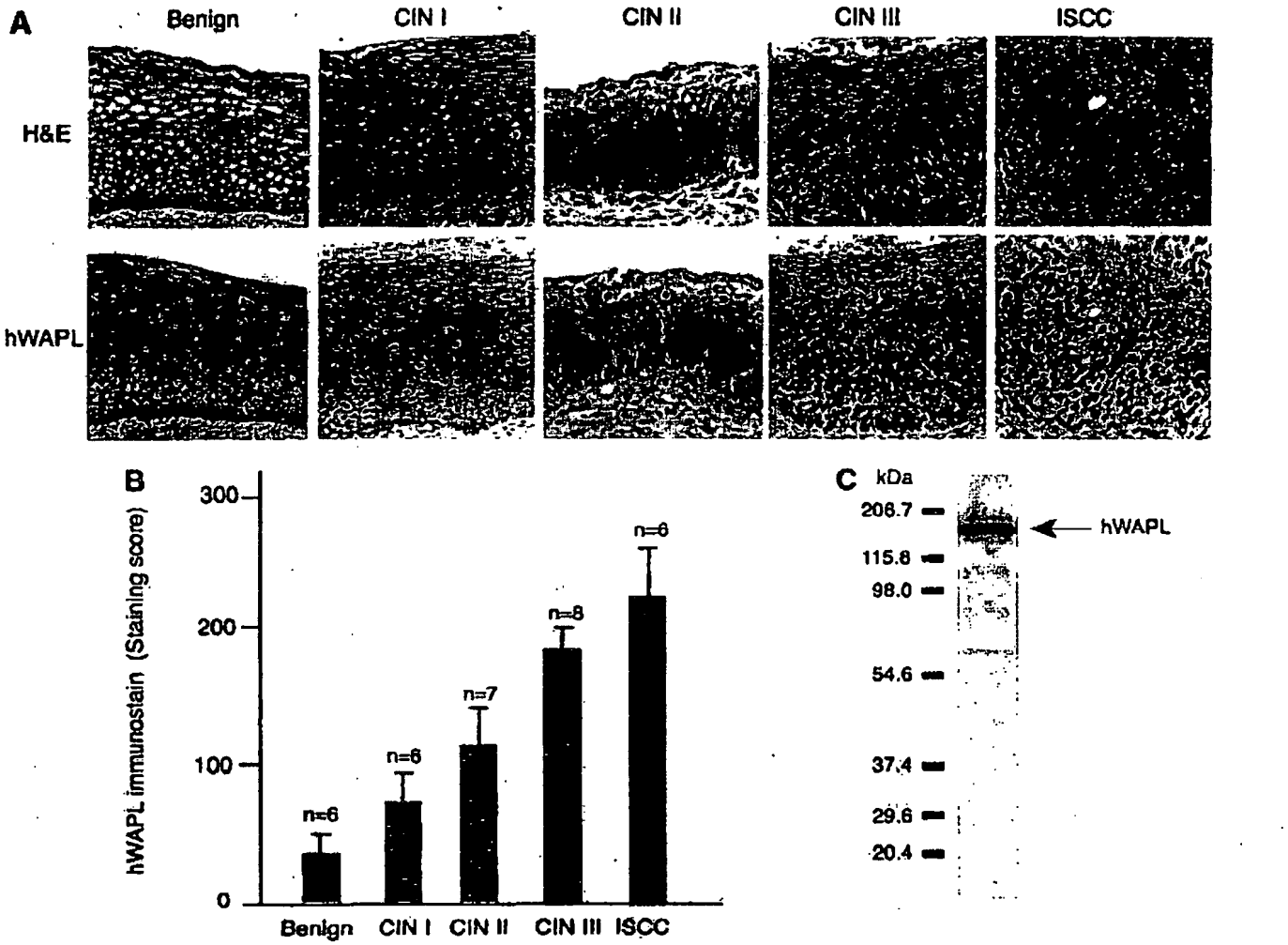

Fig. 2. Immunohistochemical analysis of human wings apart-like (*hWAPL*) expression in uterine cervical epithelia of normal, dysplasia, and carcinoma. *A*, immunohistochemical staining of hWAPL expression in benign squamous epithelium, various grades of squamous dysplasia [cervical intraepithelial neoplasia (*CIN*) grades I, II, and III], and invasive squamous cell carcinoma (*ISCC*). hWAPL was stained with hematoxylin counterstain; H&E. *B*, graphical representation of the increase of the hWAPL expression with increasing severity of dysplasia in cervical squamous epithelia. The mean hWAPL staining scores were calculated as described (9). *Bars*, SD. *C*, Western blot analysis with the total extract from a uterine cervical cancer-derived cell line, SiHa, to confirm the specificity of the anti-hWAPL monoclonal antibody hWAPL-C.

and 4*A*). We examined various human cancer-derived cell lines and found that cervical cancer-derived cell lines containing both HPV-positive and -negative cells exhibited higher levels of hWAPL expression compared with the other cell lines (data not shown). Then, we examined the effects of suppressing *hWAPL* in a cervical cancer-derived cell line, SiHa. siRNA transfection at a concentration of either 1 nM siRNA(I) or siRNA(II) reduced *hWAPL* mRNA levels (Fig. 4*B*). siRNA(I) was more effective at reducing *hWAPL* mRNA than siRNA(II). Thus, we used siRNA(I) in the subsequent experiments. hWAPL protein levels were also significantly reduced after siRNA(I) transfection (Fig. 4*C*). Interestingly, siRNA(I) repressed the growth of the cells and subsequently induced cell death (Fig. 4, *D* and *E*). siRNA(II) repressed cell growth in a similar manner as siRNA(I) (Fig. 4*D*), suggesting that the effects of these siRNAs on proliferation and viability are likely caused by the repression of *hWAPL* expression. Similar results were obtained in another cervical cancer-derived cell line, CaSki, with 10 nM siRNA(I) (data not shown). On the contrary, we did not observe any effects of siRNA(I) on cells expressing relatively low levels of *hWAPL*, such as Saos-2 and HCT116 (data not shown).

To investigate the fate of cells transfected with siRNA(I), we analyzed siRNA-transfected cells by flow cytometry (Fig. 5). In siRNA(I)-transfected cells, the population of cells exhibiting S phase DNA content increased (Fig. 5; 48 and 72 h). In addition, there was an increase in the number of apoptotic cells exhibiting subG$_1$ DNA content (Fig. 5; 72 h). Many cells showing S phase DNA content may also be apoptotic cells at G$_2$-M phase. Taken together, these results suggest that a malfunction in the hWAPL pathway activates an S phase checkpoint or another apoptotic pathway and consequently leads to cell death.

DISCUSSION

In this study, we report the isolation and characterization of a novel human gene termed *hWAPL*. We were unable to identify additional genes similar to *wapl* within the human genome sequence database. Thus, although the high-sequence conservation between hWAPL and wapl is limited to a third of the protein sequence encoded by *wapl* (Fig. 1*A*), we consider hWAPL to be the human homologue of wapl. We did not find any protein sequence motifs in hWAPL, except for the WAPL-conserved region (Fig. 1*A*). We therefore expect that hWAPL has similar functions to the wapl protein. Two hybridization signals for *hWAPL* were visible by Northern blot analysis (Fig. 1*B*). Western blot analysis, however, detected only a single band for

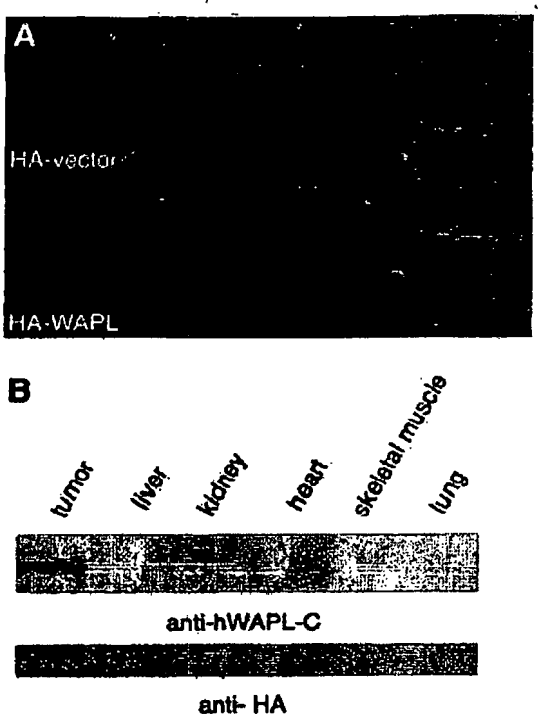

Fig. 3. Human wings apart-like (*hWAPL*) overexpression promotes tumor development. *A*, tumorigenecity of HA-hWAPL 3T3 in nude mice. The lower mouse in the *panel* is shown 10 days after the injection of HA-hWAPL-3T3 at three s.c. sites. The upper mouse was injected with the control HA-3T3 cells. *B*, Western blot analysis of hWAPL protein in tumor and other control tissues from HA-hWAPL-3T3-injected nude mice. *Top panel*, anti-hWAPL antibody; *bottom panel*, anti-HA antibody.

hWAPL (Fig. 2*C*). In addition, we did not obtain additional nucleotide sequences similar to the open reading frame of *hWAPL* by PCR analysis with various PCR primers (data not shown). Thus, we consider that the two hybridization signals may reflect the difference of the length of the untranslated regions of the *hWAPL* mRNA.

High-level expression of *hWAPL* was observed in cervical cancers (Fig. 1, *B* and *C*). Furthermore, hWAPL-overexpressing 3T3 cells developed into tumors on injection into nude mice (Fig. 3). These results suggest that *hWAPL* has oncogenic characteristics. Cervical cancer is a serious health problem, with ~500,000 women developing the disease each year worldwide. In many developing countries, it is the most common cause of cancer death and years of life lost because of cancer (20). Although the fundamental role of high-risk HPV infection in the pathogenesis of cervical carcinoma is well established, other factors are thought to play a role in cervical carcinogenesis (8, 21). Because all of uterine cervical samples examined were HPV positive (data not shown), it is still to be confirmed whether hWAPL expression is inducible by HPV infection. However, HPV-positive normal cervical tissue samples exhibited low hWAPL expression (Fig. 1, *B* and *C* and data not shown), and an HPV-negative, uterine cervical cancer-derived cell line, C33A, showed high hWAPL expression (data not shown). Thus, hWAPL expression is likely to be more closely related with cervical carcinogenesis than HPV infection. Recently, Acs *et al.* (9) found significant correlation among expression of Epo receptor, $p16^{INK4a}$, and *bcl*-2 in benign and dysplastic squamous epithelia. In our results, hWAPL showed similar expression pattern to Epo receptor and $p16^{INK4a}$ in benign and dysplastic cervical squamous epithelia and invasive squamous cell carcinomas (Fig. 2, *A* and *B*). Although we did not find any evidence for hWAPL being involved in hypoxia-inducible Epo signaling, hWAPL may cooperate with the Epo signaling in the progression of cervical neoplasia. These observations indicate that hWAPL overexpression can be used as a useful diagnostic tool in the detection of cervical dysplasia like $p16^{INK4a}$ (22) and Epo receptor (9). In addition, our results provide the necessity to investigate the potential of hWAPL as a cancer therapeutic target.

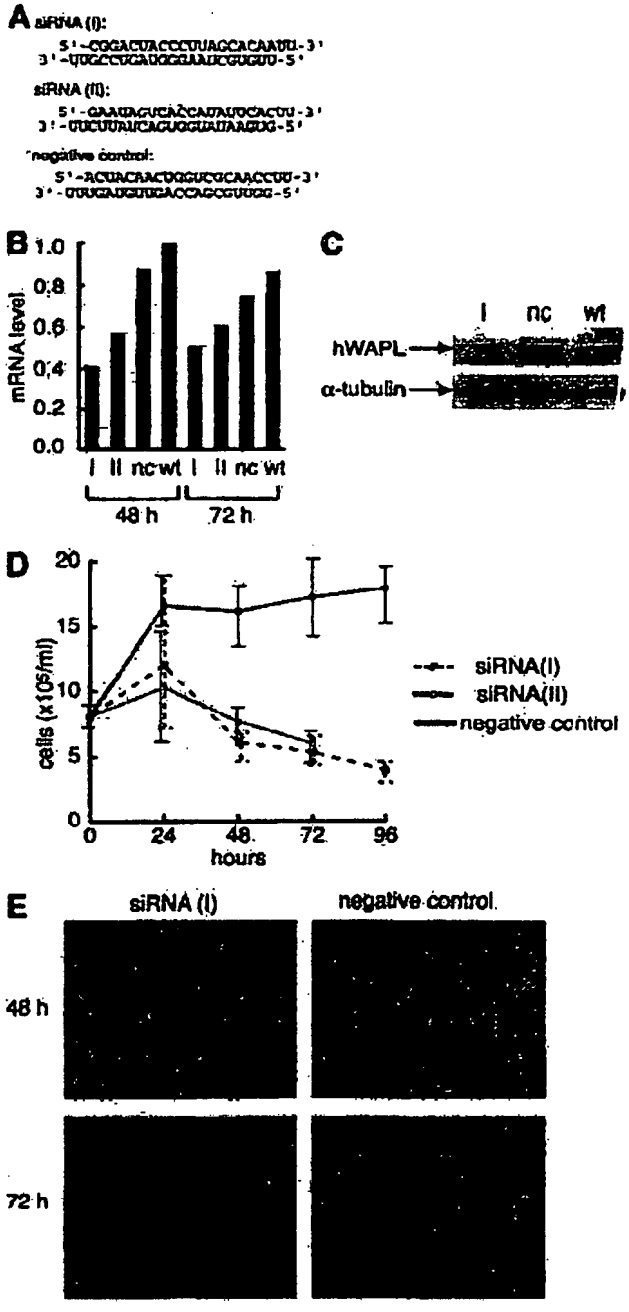

Fig. 4. Repression of human wings apart-like (*hWAPL*) expression by small interfering RNA (*siRNA*) treatment induces cell death. *A*, sequences and structures of siRNAs. The negative control siRNA possesses the same nucleotide composition as siRNA(I) but lacks homology to any known human genes. *B*, reduction of the *hWAPL* transcript by siRNA in SiHa cells. After siRNA transfection, SiHa cells were harvested at either 48 or 72 h. Total RNA was extracted from the cells and subjected to real-time PCR analysis. *I*, siRNA(I); *II*, siRNA(II); *nc*, negative control siRNA; *wt*, untransfected wild type. Data were normalized to a maximum mRNA level that was arbitrarily set to 1 in the graphical presentation. *C*, reduction of hWAPL protein levels by siRNA. Western blot analysis of total cell extracts from untreated SiHa or SiHa cells 72 h after transfection with siRNA(I) or negative control siRNA. α-tubulin is shown as a loading control. *D*, active siRNA specific for *hWAPL* induces cell death. SiHa cells transfected with siRNA(I), siRNA(II), or negative control siRNA were harvested at 24, 48, 72, and 96 h after transfection. Cell numbers were counted using an erythrometer. *Bars*, SE. *E*, representative phase-contrast images of SiHa cells transfected with siRNA(I) and negative control siRNA are shown.

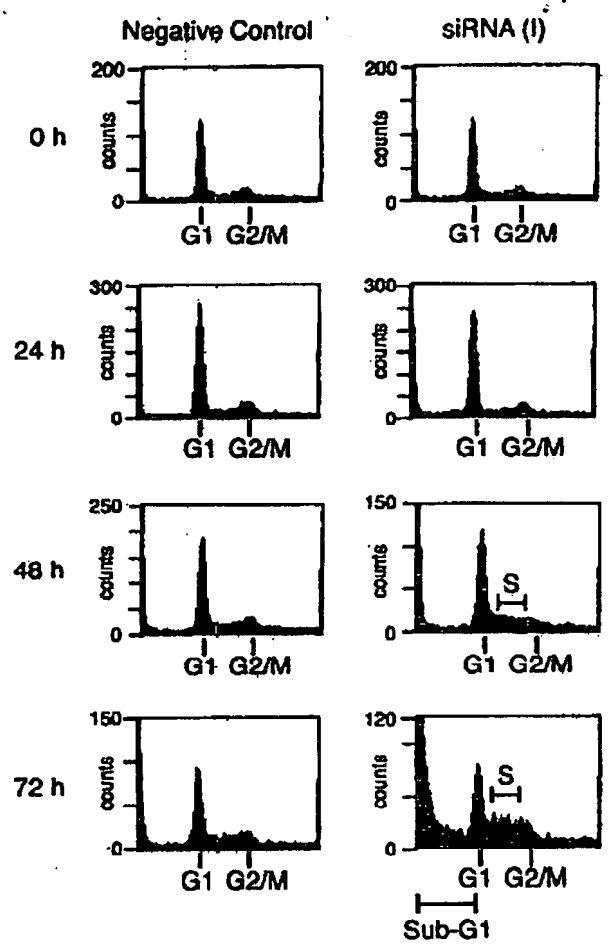

Fig. 5. Flow cytometric analysis of SiHa cells after small interfering RNA (*siRNA*) transfection. SiHa cells were transfected with either siRNA(I) or negative control siRNA, then harvested at 24, 48, and 72 h after transfection. Cells were stained with propidium iodide and subjected to flow cytometric analysis to examine DNA content. A total of 50,000 cells was counted for the sample siRNA(I) 72 h, and 20,000 cells were counted for the other samples.

Loss of WAPL was embryonic lethal in mouse (data not shown), and repression of hWAPL expression in SiHa cells led to cell death (Fig. 4). Flow cytometry analysis demonstrated that malfunction of hWAPL may cause apoptosis and/or arrest of cells at S phase (Fig. 5). In addition, *Drosophila wapl* is associated with regulation of chromatin organization (1). Thus, we expect that hWAPL is also associated with regulation of chromatin structure, and deregulation of hWAPL expression may induce chromosomal instability. Although additional investigations are necessary to elucidate the actual function of hWAPL in normal and malignant cells, our results have demonstrated that the novel oncogene, *hWAPL*, is one of the essential genes for development and cell growth and may play a significant role for cervical carcionogenesis and tumor progression.

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